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Metabolic compensation of steroidal glycoalkaloid biosynthesis in transgenic potato tubers: using reverse genetics to confirm the in vivo enzyme function of a steroidal alkaloid galactosyltransferase☆

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Abstract

Steroidal glycoalkaloids (SGAs) are secondary metabolites of Solanaceous plants. Two predominant glycoalkaloids, α-chaconine and α-solanine are produced in potatoes. An antisense transgene was constructed to down-regulate glycoalkaloid biosynthesis using a potato cDNA encoding a sterol alkaloid glycosyltransferase (*Sgt1*). Introduction of this construct into potatoes resulted in some lines with an almost complete inhibition of α-solanine accumulation. This inhibition was compensated by elevated levels of α-chaconine and resulted in wild type total SGA levels in the transgenic lines. In vitro assays with the recombinant SGT1 isolated from yeast demonstrated that *Sgt1* encodes an enzyme capable of both glucosyltransferase and galactosyltransferase activity with a preference for UDP-galactose as the sugar donor. Together this data confirms SGT1's role in vivo as the solanidine:UDP-galactose galactosyltransferase.

Keywords: Antisense; Chaconine; Galactosyl transferase; Secondary metabolism; Solanine; Transgene

1. Introduction

The steroidal glycoalkaloids (SGAs) are a family of secondary metabolites produced by Solanaceous plants, including potato, tomato and eggplant. These compounds, proposed to contribute to defense from insect and/or microbial pests [1,2], present quality (flavor) and potential food safety concerns if present at excessive levels. Potential safety issues have led to the establishment of guidelines limiting SGA levels in released commercial potato cultivars [1]. Potato SGAs contain either glucose (α-chaconine) or galactose (α-solanine) as the primary glycosyl residue (Fig. 1a). The enzyme solanidine UDP-glucose glucosyltransferase catalyzes the biosynthesis of γ-chaconine from UDP-glucose and solanidine [3–6]. The biosynthesis of γ-solanine involves a distinct galactosyltransferase [3,5,6]. However, in crude protein extracts from potatoes galactosyltransferase activity is unstable and present at low levels relative to glucosyltransferase activity [3,4,6].

A potato cDNA for a sterol alkaloid glycosyltransferase (*Sgt1*) gene was cloned in yeast by selection of yeast with the ability to detoxify the tomato SGA solasodine in the growth medium [7]. In vitro analysis of partially purified protein from yeast expressing SGT1 showed UDP-glucose dependent solanidine glucosylation activity and reaction products [7]. Both partially purified SGT1 from potato and the recombinant protein from yeast were more active with

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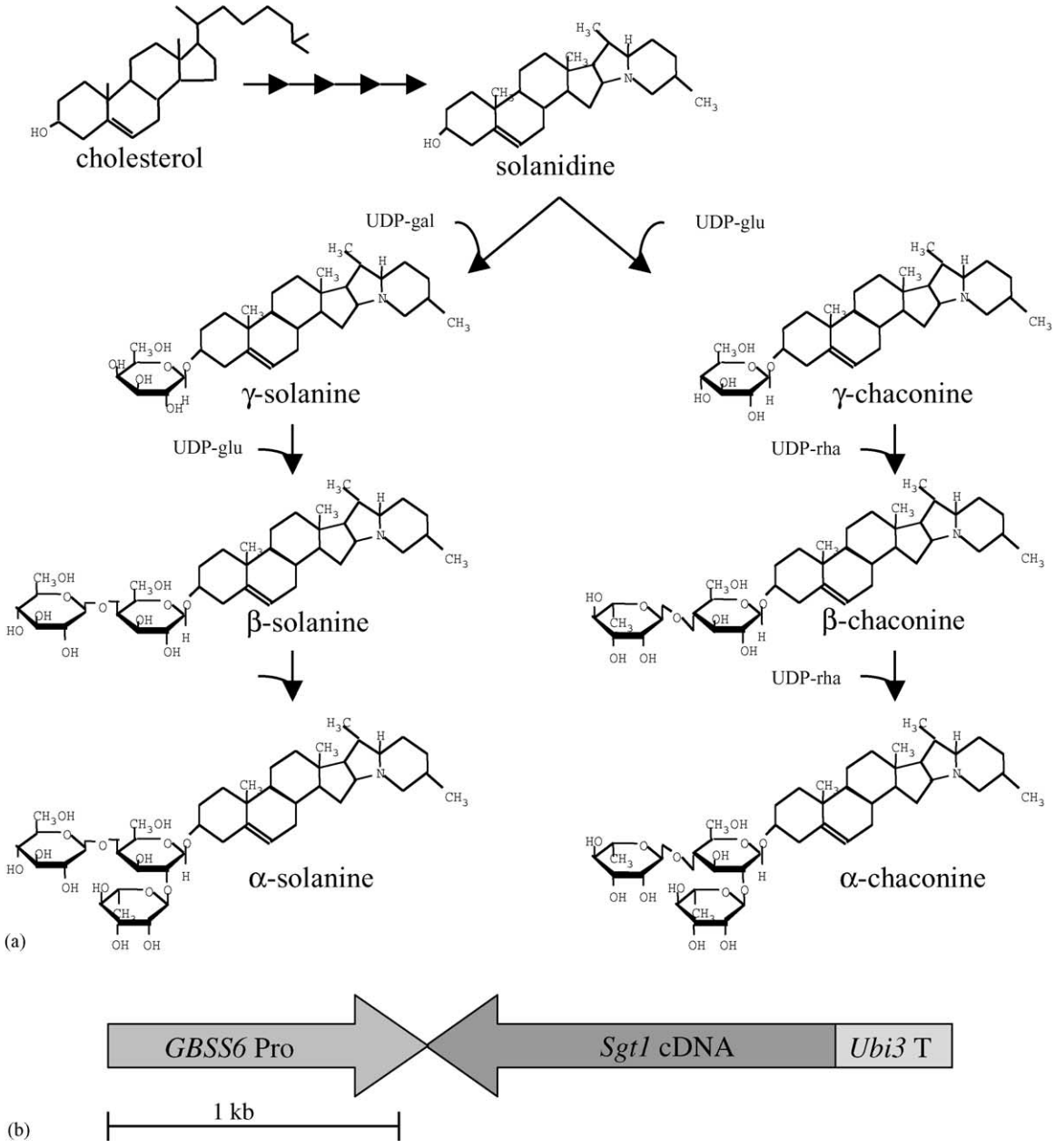

Fig. 1. (a) The SGA biosynthetic pathway. Proposed pathway for synthesis of the two predominant potato glycoalkaloids from the aglycone, solanidine. (b) Schematic representation of the *Sgt1* antisense construct under control of the *GBSS6* promoter and *Ubi3* terminator.

steroidal alkaloid aglycones from tomato, solasodine and tomatidine, than with the potato aglycone solanidine [4,7].

Transgenic lines were constructed using the *Sgt1* coding sequence in the antisense orientation [8] driven by either the constitutive cauliflower mosaic virus (*CaMV*) *35S* promoter or the tuber-specific granule bound starch synthase (*GBSS6*) [9] promoter. Given the direct relationship between *Sgt1* transcript and protein levels [7], down-regulation of this gene was predicted to result in lower total SGA levels. However, transgenic isolates had total SGA levels that varied from 30% greater than normal to 40% lower than normal. In the first year field trials planted from greenhouse-grown minitubers, large standard deviations of the means were observed and no statistically significant differences were seen between controls and the transgenic population. In the second and third years, only one of the 13 lines showed a statistically significant reduction in total SGAs [8]. In all 13 lines, antisense transcripts were easily detected, but there was little correlation between *Sgt1* transcript and total SGA levels; suggesting that the lower SGA levels in these lines was due to somaclonal variation rather than targeted down-regulation of *Sgt1* [8]. Here we report the generation of additional *Sgt1* antisense lines exhibiting novel chemotypes based on analysis of the component glycoalkaloids α-chaconine and α-solanine and the activity of recombinant SGT1.

2. Materials and methods

2.1. Plant materials

Potato (*Solanum tuberosum* L.) cv. Lenape [10] was grown in the glasshouse in Albany, CA for collection of meristems for DNA isolation. For SGA and RNA analyses, Lenape tubers were harvested from replicated field plots [11] in Aberdeen, ID, USA and Desiree tubers were harvested from glasshouse-grown plants in Invergowrie, Dundee, UK.

2.2. Transgene construction

The antisense transgene was constructed with the 1571 bp *Sgt1* cDNA sequence (GenBank accession no. U82367) [7] in antisense orientation driven by the 1206 bp potato *GBSS6* promoter [12], for tuber-specific transcription [9], followed by a 404 bp potato *Ubi3* polyadenylation signal [13] (Fig. 1b). The transgene was transferred to a modified pBINPLUS binary vector [14], pBINPLUS/ARS for mobilization into potato varieties Lenape and Desiree via *Agrobacterium*-mediated transformation [15]. The pBINPLUS/ARS vector is identical to pBINPLUS except that control sequences for the *NptII* plant selectable marker (nopaline synthase promoter and terminator pBINPLUS) have been replaced by potato *Ubi3* promoter and polyadenylation signal [13] sequences in pBINPLUS/ARS.

2.3. Expression vector construction

For expression of SGT1 protein in yeast, the longest open reading frame (ORF) in *Sgt1* was amplified directly from an *S. tuberosum* cv. Lemhi cDNA library [16] by PCR using primers containing a KpnI site added to the 5′ end and an XhoI site added to the 3′ end of the ORF. The XhoI site replaced the native stop codon creating a read through fusion for expression of a 6xHis tag in the pYES2.1/V5-His TOPO cloning vector (Invitrogen). Yeast was transformed with the recombinant vector, grown under inducing conditions and protein extracted according to manufacturer's recommendations (Invitrogen). Protein purification was carried out using the His protein isolation system (Sigma) according to manufacturers instructions. Purification was assessed via SDS-PAGE and staining with Coomassie blue. Elution of recombinant proteins was monitored by western blot analysis using the antiV5 epitope antibody (Invitrogen). Solanidine glycosyltransferase assays were carried out using 100 μL of column eluate after buffer exchange and concentration. Assays were run for 60 min at 37 °C. SGAs and UDP-sugars were provided at 33 μM. Radioactive UDP-sugar stock solutions were prepared to contain ∼5 × 10^5 dpm UDP-[^{3}H]-glucose or UDP-[^{3}H]-galactose per reaction. Inhibitor studies included the addition of α-solanine or α-chaconine dissolved in DMSO to the final concentrations as indicated corrected for the control reactions with the addition of an equal volume of DMSO in the absence of inhibitor. Values presented represent the mean of duplicate assays. Separation of the product using anion exchange resin was as previously described [4].

2.4. Steroidal glycoalkaloid determinations

Levels of SGAs were quantified from slices of tubers or whole minitubers of field-grown Lenape or glasshouse-grown Desiree, respectively. Field-grown tubers were cut in half longitudinally through the widest dimension ∼2.5 mm off center with an 21 cm chef knife and the central longitudinal section of 5 mm was cut with a mandolin. Sections were frozen in liquid nitrogen, freeze dried, milled and the dry powder extracted and analyzed for SGAs by HPLC as described by Hellenaes [17].

2.5. RNA blots

Total RNA was prepared from tuber peels obtained using a hand-held vegetable peeler. Peels were frozen in liquid nitrogen, ground and extracted for RNA as previously described [18]. Poly(A)$^+$ RNA was obtained using magnetic beads according to manufacturers instructions (Promega Z5300) fractionated by agarose gel electrophoresis, and transferred to a nylon membrane (Roche) [19]. RNA blots were hybridized with a random primed (GE Healthcare) double stranded probe of the *Sgt1* cDNA fragment [7] or a patatin storage protein cDNA [20]. For detection of antisense specific transcripts, a digoxigenin-labeled strand-specific RNA probe was generated using the T7 primer in the sense direction of the *Sgt1* cDNA subcloned into pBluescriptII SK(-) (Stratagene) using the Dig RNA Labelling Kit (Roche) according to manufacturers instructions.

2.6. DNA blots

DNA was isolated from young shoot tips frozen in liquid nitrogen and extracted as previously described [21]. DNA was digested with restriction enzymes BamHI and HinDIII, separated by agarose electrophoresis, blotted to charged nylon membranes, and hybridized with a double stranded *NptII* probe isolated from a PmeI digest of pBINPLUS/ARS.

3. Results

3.1. Steroidal glycoalkaloid accumulation

The SGA levels were measured in uniform slices of field-grown tubers or whole glasshouse-grown minitubers in 2001. Fig. 2 shows the amount of component alkaloids α-solanine and α-chaconine in the transgenic Lenape and Desiree lines. The plant lines are sorted by descending total SGA levels (the sum of α-solanine and α-chaconine). The range in average total SGA levels from the tubers for the

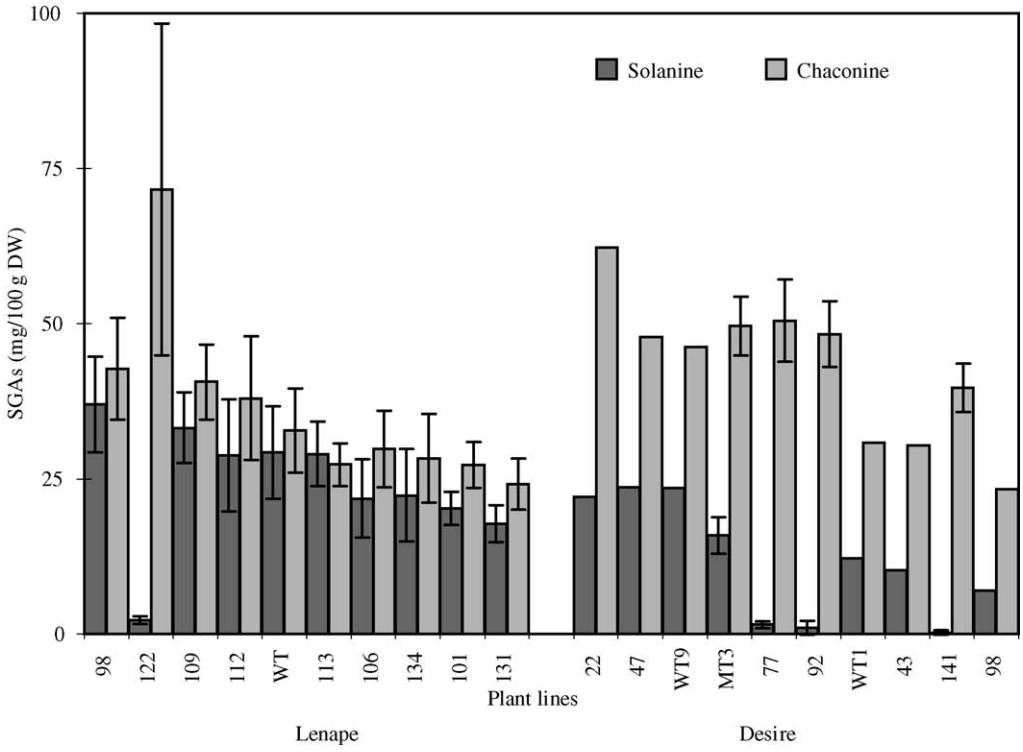

Fig. 2. SGA content of tubers from transgenic potatoes expressing the *Sgt1* antisense transgene. Total SGA levels showing the range of alkaloids accumulated in selected transgenic, wild type (WT) and empty vector (MT) control lines of Lenape and Desiree. Values represent average and standard error for triplicate samples from triplicate plots (Lenape $n = 9$) and single or triplicate tuber samples (Desiree $n = 3$) WT and MT.

Sgt1 antisense Lenape lines varies from 29% above to 32% below wild type. Analysis of variance for total SGA levels indicates that total SGA levels are only significantly lower than WT in Lenape line 131 ($P < 0.001$). The variation for Desiree lines was slightly larger (49% higher to 46% lower than the average of WT controls) but many of the data points represent un-replicated samples. Among the triplicate analyses, total SGAs are only significantly lower in line 141 versus MT3.

Analysis of variance for α-solanine levels shows that the reduction in Lenape line 122 is significantly different from all other lines and that α-solanine levels in the remaining lines are not significantly different than WT. In Desiree lines 77, 92 and 141 the reductions of α-solanine levels are significantly different from the empty vector control (MT3). The number of lines with dramatically decreased solanine levels is consistent with the low (5–10%) efficiency generally observed in transgenic antisense potato populations [11].

In Lenape line 122, with a low level of α-solanine, the α-chaconine content was elevated, and there was essentially no change in total SGA levels relative to the WT. Analysis of variance for α-chaconine levels showed that the increase in Lenape line 122 was significantly different from all other lines. Among the remaining Lenape lines only the highest and lowest α-chaconine levels were significantly different from each other (lines 98 and 131). In Desiree, the modest increases of α-chaconine in lines 77, 92 and 141 were not significantly different than controls. To understand the relationship of the antisense transgene on altered α-solanine levels an analysis of mRNA abundance was performed.

3.2. Expression of Sgt1

The steady state levels of *Sgt1* mRNA were examined by RNA blot analysis. To test for transgene efficacy in down-regulating *Sgt1* expression, poly(A)$^+$ RNA was isolated from transgenic lines and probed with the *Sgt1* sequence (Fig. 3a). A range of transcript levels was observed: nearly undetectable at the predicted size in line 122, low in the WT tubers, and very strong in line 101. To explore the contribution of antisense *Sgt1* transcript to the steady state levels observed in Fig. 3a, a sister blot was hybridized to a non-radioactive antisense specific probe (Fig. 3b). Antisense transcripts are absent in the WT control as expected, weak in line 112, and missing at the expected position in 122. The RNA blot in Fig. 3a was re-hybridized with a probe for the storage protein patatin mRNA (Fig. 3c). This probe reveals strong bands in all lines indicative of RNA relatively free from degradation and consistently loaded across the lanes. To examine the effect of transgene integration on antisense action, genomic DNA was examined for *Sgt1* antisense transgene integration patterns.

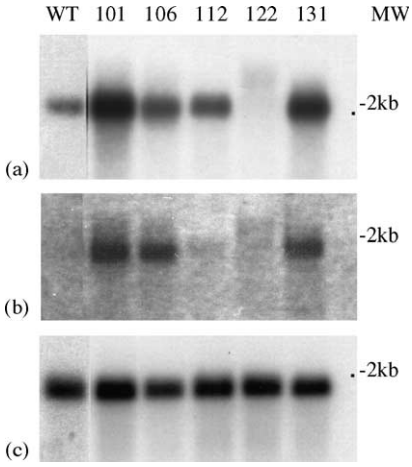

Fig. 3. *Sgt1* transcripts in transgenic Lenape potato tubers. Poly(A)$^+$ RNA from tubers of Lenape clones transformed with the *Sgt1* antisense transgene was probed with: (a) double stranded *Sgt1* probe, (b) digoxygenin labeled singled stranded antisense transcript probe, and (c) the patatin storage protein transcript.

3.3. Analysis of transgene integration

An examination of the integration patterns in select transgenic Lenape lines was carried out by genomic DNA Southern blot analysis. The results indicate a simple pattern suggesting one or two insertions for most lines (Fig. 4). However in line 122, which was lacking *Sgt1* transcripts, a complex pattern is observed suggesting multiple insertions.

3.4. Biochemical assay or recombinant SGT1 protein

The previously described in vitro activity of recombinant SGT1 as a solanidine glucosyltransferase predicted a reduction of α-chaconine in antisense lines with effective down regulation. Due to the unstable galactosyltransferase activity in potato extracts [4] the partially purified recombinant enzyme was not tested for its ability to galactosylate solanidine [7]. The surprising observation that the major affect of antisense *Sgt1* was to reduce α-solanine levels indicated a need for further examination of the recombinant SGT1 protein activity. The new recombinant SGT1 was tested against Solanaceous SGAs using either UDP-[^{3}H]-glucose or UDP-[^{3}H]-galactose as the sugar donor. The results are summarized in Table 1. While the recombinant SGT1 is capable of utilizing UDP-glucose as a substrate in vitro, this activity is drastically less than the activity observed with UDP-galactose. The activity with the tomato SGAs is still higher than with the native solanidine as substrate regardless of the sugar donor. In a separate experiment (for an enzyme preparation with activity of 3.9 nmol min^{-1} mg^{-1}) the addition of 100 μM α-solanine or α-chaconine caused a 58 and 65% reduction, respectively, in SGT1 solanidine:galactosyltransferase activity. This demonstrates feedback inhibition of the primary galactosylation step by both downstream triose end products.

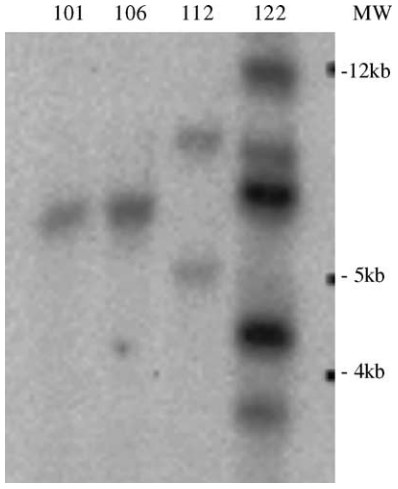

Fig. 4. Integration pattern of *Sgt1* transgenes in selected Lenape potato lines. DNA from leaf tissue of Lenape clones transformed with the *Sgt1* antisense construct was probed with the *NptII* gene.

4. Discussion

Antisense transgenes have been successfully employed to down-regulate target genes in potato [22,23]. Effective down-regulation of SGT1 resulting in a decrease in total SGA levels was expected in 5–10% of the antisense RNA-expressing lines [11]. Over 50 lines developed using the *Sgt1* construct were previously analyzed and a continuum of total SGA levels observed [8] without a decrease of more than 40%. This variation was within the range observed by others [24] and is now attributable to somaclonal variation.

If *Sgt1* encodes an enzyme with solanidine glucosyltransferase activity, down-regulation should result in a specific reduction in α-chaconine (Fig. 1), the more abundant of the two major SGAs that accumulate in potato. This could be hoped to cause a reduction of over 50% in the levels of total SGAs. Some of the newly generated *Sgt1* antisense lines have dramatically reduced accumulation of α-solanine associated with a range of increases in α-chaconine. Down-regulation of *Sgt1* results in a dramatic shift in glycoalkaloid profiles with little or no reduction in total levels of these undesirable metabolites.

Examination of mRNA for all *Sgt1* transcripts reveals a wide range of expression (Fig. 3a). The signal is low as expected in non-wounded WT tubers, absent in line 122, and high in lines 101 and 131. Analysis of the mRNA with an antisense specific probe reveals that the majority of the variation is due to varying levels of the antisense transcript (Fig. 3b). This is similar to that observed in the total RNA of early transgenic lines where the contribution of the *35S* promoter/nopaline synthase (*Nos*) terminator construct showed higher levels of antisense expression than the *GBSS6/Nos* construct, but, there was no correlation with total SGA levels [8]. However, line 122 is lacking *Sgt1*

Table 1
Substrate preferences for recombinant SGT1

SGA substrate	UDP-glucose (nmol min^{-1} mg^{-1})	UDP-galactose (nmol min^{-1} mg^{-1})
Solanidine	ND	6.4
Solasodine	2.7	16.8
Tomatidine	3.0	20.9

Glycosyltransferase activity of the recombinant SGT1:his fusion protein expressed in yeast and purified on nickel affinity column using UDP-[^{3}H]-glucose or UDP-[^{3}H]-galactose as sugar donors, and solanidine, solasodine and tomatidine as steroid glycoalkaloid receptors. Values represent the mean of duplicate assays corrected for the control reaction using DMSO in the absence of aglycone. ND indicates no detectable activity above background (<0.25 nmol min^{-1} mg^{-1}).

transcript at the appropriate molecular weight, indicating an effective antisense transgene where both sense and antisense message are quickly degraded preventing target protein expression [25]. This is associated with a significant reduction in α-solanine and increase in α-chaconine levels.

Examination of the transgene integration patterns reveals one or two bands in all of the Lenape lines as was observed for the early transgenic lines [8] with the exception of line 122. The complex integration pattern in transgenic plant line 122 is associated with an absence of *Sgt1* transcripts at the appropriate size. Complex integration patterns have been associated with effective antisense down regulation in other systems [26].

Taken together, the complex integration, the lack of sense transcripts and the dramatic shift in SGA profiles leads us to conclude that in plant line 122 we are exposing the true function of SGT1. As opposed to reducing accumulation of α-chaconine as originally predicted, the major effect is to reduce α-solanine accumulation. The in vitro data establish the ability of this enzyme to function as both a glucosyltransferase [7] and a galactosyltransferase (Table 1). The SGT1 enzyme shows greater activity with UDP-galactose as the sugar donor for all the SGA aglycones tested and higher activities with the tomato SGAs. The data presented in Table 1 indicates that the previously demonstrated SGT1 activity in vitro [7] does not represent the primary function of SGT1 in glycoalkaloid biosynthesis in potato. We have demonstrated the preference of SGT1 for UDP-galactose over UDP-glucose in vitro, and the data from antisense lines establishes the in vivo function of SGT1 as a galactosyltransferase specific for the synthesis of β-solanine (solanidine:UDP-galactose 1 → 3-*O*-β-D-galactosyltransferase). This leaves an as yet unidentified gene encoding the enzyme responsible for solanidine glucosylation in vivo.

Metabolic compensation in alkaloid biosynthesis has been observed in other Solanaceous species either by antisense [27] or chemical inhibition [28]. Lenape line 122 shows essentially complete compensation with a 92% reduction in α-solanine associated with a 118% increase in α-chaconine compared to WT levels. The compensation in Desiree lines is not as complete with 91–99% reduction in α-solanine and only a 3–31% increase in α-chaconine levels. These increases in α-chaconine are not statistically significant in the current samples and Desiree line 141 showed a significant decrease in total SGAs as a result of reduced α-solanine. Compensation by over accumulation of α-chaconine when α-solanine accumulation is inhibited has now been observed in additional Desiree and Lenape antisense *Sgt1* lines. Examination of SGA biosynthetic intermediates in these lines by HPLC–MS (data not shown) indicates no significant change in the accumulation of α-solanine or α-chaconine intermediates. The compensation in potato SGA metabolism is dramatic. While total levels of SGAs remained essentially unchanged the bulk of the accumulated end product was the more toxic α-chaconine at the expense of α-solanine. The metabolic compensation data is consistent with feedback regulation by total SGAs on their biosynthesis, particularly in Lenape, at either of the solanidine glycosyl transferases, the branch point in the pathway, or by some upstream intermediate in the biosynthesis of solanidine from cholesterol. The addition of either α-solanine or α-chaconine resulted in over 50% inhibition of SGT1 activity suggesting that accumulation of triose end products may be regulated by feedback inhibition at the primary glycosylation step. A full understanding of the regulation of this pathway will require the isolation of additional enzymes and further experimentation.

These results emphasize the need for in vivo/in planta analysis for the appropriate assignment of metabolic function of a specific gene sequence. This is especially true in complex secondary metabolic pathways where several enzymatic reactions share similar substrates. The reverse genetic strategy employed here allowed assignment of the *Sgt1* gene product in glycoalkaloid biosynthesis and dictated the need for appropriate studies of biochemical function in vitro. The identification of the true in vivo solanidine galactosyltransferase and the production of stable recombinant protein will provide valuable tools for further study of this important biosynthetic pathway. Finally, these data indicate the importance of considering kinetic features when designing transgenes to control flux through complex multi-enzyme pathways.

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